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Test system for the recognition ~~of different markers,~~ its preparation and use

The present invention relates to a test system comprising at least two recognition species which recognize at least two different markers with formation of a complex, its preparation and use in a suitable detection process.

The areas of application of test systems, such as diagnostics, are widespread in biology, biochemistry, medicine and pharmacology. Especially in medicine, a reliable and clear diagnosis of diseases, such as viral infections or cancer, is of extreme importance for increasing the quality of life, since only by early recognition of a disease can a timely and effective treatment take place. Based on the recognition of disease-specific markers or ligands, such as nucleic acid sequences, proteins or antigens, the pathogen or the disease in the biological sample is detected. Diagnostic tests are widespread in which a marker or a class of markers in each case is detected, such as in ELISA or in amplification methods, such as PCR, b-DNA, Southern, Western or Northern blotting. The types of detection used range from simple staining methods and calorimetric methods via fluorescence energy transfer (FRET) and fluorescence quenching up to scintillation proximity assay (SPA).

A significant disadvantage in the use of only one marker or one class of marker is that false-positive test results easily result, which also lead to wrong conclusions regarding a specific disease. A second test or still further tests must often therefore be carried out on the same or complementary analytes in order to be able to make a reliable statement with regard to sickness/health. This leads to more tests, whose results are to be compared with one another, which is at the same time laborious and cost-intensive.

It is therefore an object of the present invention to develop qualitatively better, less complicated and less expensive analyte tests than those already known.

Surprisingly, it has now been found that the linkage of two or more test results at the molecular level in the sense of Boolean linkage allows a qualitatively very good, simple and inexpensive analyte test, the different test results essentially not interfering with one another.

The invention therefore relates to a detection process comprising the following steps:

(a) treatment of a sample comprising a first and a second marker with a first recognition species which recognizes the first marker,
(b) treatment of the sample with a second recognition species which recognizes both the first marker and the second marker,
(c) treatment of the sample with a third recognition species which recognizes the second marker,
(d) detection of the presence or absence of a complex of the recognition species and markers mentioned.

In addition, the present invention relates to a detection process comprising the following steps:

(a) treatment of a sample comprising a first and a second marker with a first recognition species which recognizes the first marker,
(b) treatment of the sample with a second recognition species which recognizes the first marker and a third recognition species,
(c) treatment of the sample with a third recognition species which recognizes the second marker and the second recognition species,
(d) detection of the presence or absence of a complex of the recognition species and markers mentioned.

To increase the specificity, it is advantageous in a further embodiment that further recognition species which recognize further markers are employed in further treatment steps, which results in n + 1 recognition species on widening to n ligands where n is equal to a natural number.

In further preferred embodiments, the detection process is carried out in homogenous, partly homogeneous (modular) or immobilized form. In a homogeneous embodiment, the binding events are produced stepwise and the complex possibly formed is detected in a "proximity assay". The first and the last components are preferably labelled such that they can only produce a signal when mutually opposite. Preferred detection processes are, for example, LOCI (Ullmann, E. et al. (1994) Proc. Natl. Acad. Sci. USA 91, 5426), fluorescence energy transfer (FRET) Cardullo, R.A. (1992) in "Nonradioactive Labeling and Detection of Biomolecules", 414-423, Springer Verlag), fluorescence quenching (Ladokhin, A.S. (1997) "Distribution analysis of depth-dependent fluorescence quenching in membranes: a practical guide", Methods Enzymol., 278, 462-473) or scintillation proximity assay (SPA) (Picardo, M. & Hughes, K.T. (1997) "Scintillation proximity assays. High Throughput Screening", Ed. Devlin, J.P.; Verlag Dekker, New York, N.Y., 307-316).

In a partly homogeneous or modular embodiment, the binding events are produced stepwise and in solution and, as soon as the complex has formed, it is bound to a solid support via one of the components. The complex formed is detected by means of a marker, in particular a non-radioactive marker or radioactive marker, preferably by means of a fluorescence marker, enzymatic marker, redox marker or spin marker (Kessler, C. (Ed.) Nonradioactive Labeling and Detection of Biomolecules (1992), Springer Verlag, 414-423).

In an immobilized embodiment, a recognition species, preferably the first recognition species, is bound to a solid support and subsequently built up by

stepwise addition of the other components of the complex. Labelling is preferably carried out by methods which are identical or similar to those in the case of the partly homogeneous embodiment.

A suitable support for the immobilization is especially solid or gelatinous material, in particular chip material and/or thin layers of the material, preferably ceramic, metal, in particular noble metal, glasses, plastics, crystalline materials or (bio)-molecular filaments, in particular cellulose or structural proteins.

The recognition species and/or markers employed in the detection process according to the invention are, in particular, a synthetic substance, a natural substance and/or a natural substance derivative, preferably a peptide, peptoid, protein, saccharide or a nucleic acid. A receptor or a functional part thereof, for example, is particularly preferred, in particular a functional part which originates from the extracellular domain of a membrane-based receptor, an antibody or a functional part thereof, in particular an Fv fragment (Skerra & Plückthun (1988), Science 240, 1038), a single-chain Fv fragment (scFv; Bird et al. (1988), Science 242, 423; Huston et al. (1988), Proc. Natl. Acad. Sci. U.S.A. 85, 5879) or an Fab fragment (Better et al. (1988), Science 240, 1041), an aptamer, for example a DNA or RNA aptamer or derivatives thereof, for example aptamers provided with protective groups customary in nucleic acid chemistry, a cell constituent, in particular a lipid, glycoprotein, filament constituent, lectin, liposome, mitogen, antigen, secondary metabolite or hapten, a cell, in particular a lymphoid cell, or a virus, in particular a virus constituent, especially a capsid, or a viroid or a derivative, in particular an acetate, or their active parts, or a single-stranded or double-stranded nucleic acid, in particular DNA, RNA, p-RNA (Pitsch, S. et al., Helv. Chim. Acta. (1993), 76, 2161; Pitsch, S. et al., Helv. Chim. Acta, (1995), 78, 1621), p-DNA

(DE 198 37 387.2), PNA (peptide nucleic acid; Nielsen, P.E. et al. (1991) Science, 254, 1497), CNA (Aminocyclohexylnucleic acid; PCT/EP98/06002) or an aptamer (see, for example, Bock, L.C. et al. (1992) Nature, 355, 564) or hybrids of the substances mentioned.

According to the present invention, aptamers, on account of their binding properties to specific molecules which are different from nucleic acids, such as proteins, do not belong to the nucleic acids, but to antibody derivatives. DNA aptamers or RNA aptamers are preferred.

The nucleic acids according to the invention including aptamers can also be modified. For this, the methods known from nucleic acid chemistry to the person skilled in the art can be used. Modifications are preferred which lead to stabilization of the nucleic acids (see, for example, Ullmann, E. & Peyman, A. (1990) Chemical Reviews, 90, 543, No. 4).

Customarily, the recognition of a marker by a recognition species takes place by means of non-covalent interactions, in particular by means of hydrogen bonds, salt bridges, stacking, formation of metal ligands, charge-transfer complexes, Van-der-Waals forces or hydrophobic interactions. For example, a nucleic acid is recognized as a marker by a completely or partly complementary nucleic acid or a synthetic substance, such as a chemical, a natural substance and/or a natural substance derivative are recognized as antigenic substances by an appropriate antibody or antibody derivative. According to the detection process according to the invention, the markers can belong to any desired class of substance, but preferably of at least two different classes.

According to the detection process according to the invention, additionally at least one recognition species is labelled, preferably all recognition species are labelled, especially at least two recognition species are differently labelled. As already

illustrated above in greater detail, the marker, depending on whether a homogeneous, partly homogeneous (modular) or immobilized embodiment is concerned, can be non-radioactive or radioactive, preferably LOCI, FRET, fluorescence quenching, SPA, a fluorescence marker, enzymatic marker, redox marker or spin marker.

In a further preferred embodiment, the marker and/or the signal can be amplified, which leads to an increase in the sensitivity of the detection process. The amplification of the marker relates, in particular, to the amplification of nucleic acids, for example by PCR, NASBA, LCR, SDA, Qβ replication or RT-PCR (Kessler, C. (1992) supra). The signal amplification is achieved, for example, by 'cross-linking' of binding components, antibody or nucleic acid trees (e.g. b-DNA), catalytic substrate reaction (e.g. alkaline phosphatase, peroxidase, β-galactosidase) or signal cascades.

In addition to the mentioned in-vitro amplification, in-vivo amplification is also possible, e.g. detection of r-RNA, indirect detection of antigens.

In principle, the markers can be divided into two classes. In the case of 'positive markers', the absence of these markers is detected, for example, by means of the absence of a signal. Positive markers refer in general to markers present in a healthy organism, e.g. m-RNA. Negative markers are in general designated as the substances of a pathogen or of an ill organism, which can be determined by means of the detection process according to the invention .

In the detection process according to the invention, either two or more negative, two or more positive or two or more positive and negative markers can be detected. The detection thus takes place either via the occurrence or via the absence of a signal. Likewise, a displacement of a signal, e.g. by the displacement of a molecule from a complex or from its binding conformation (e.g. Molecular Beacons, S. Tyaki,

Kramer F.R., Nature Biotechnology 14, 303-308, 1996; R.P. Ekins, Clinical Chemistry, 44/9, 2015-2030, 1998) is possible in a competitive assay. For this, a substance is added to the test system which displaces one of the markers to be detected, the molecular complex built up from markers and recognition species and thus also the signal associated therewith disappearing. By means of a titration, the concentration of the displaced marker can thus be determined in a simple manner.

The detection process according to the invention can now be present in at least one of the following alternative embodiments, which are particularly preferred:

1. At least one marker is a natural or unnatural, single-stranded or double-stranded nucleic acid and each further marker is a synthetic substance, natural substance or natural substance derivative other than a nucleic acid, preferably an antigen.
2. The first marker and each further marker is a natural or unnatural, single-stranded or double-stranded nucleic acid or alternatively a synthetic substance, a natural substance or a natural substance derivative other than a nucleic acid, preferably an antigen.
3. A natural or unnatural, single-stranded or double-stranded nucleic acid as a marker is recognized by a natural or unnatural, single-stranded or double-stranded nucleic acid as recognition species.
4. A synthetic substance, a natural substance or a natural substance derivative is recognized by a synthetic substance, a natural substance or a natural substance derivative, preferably by an antibody or an antibody derivative, as recognition species.
5. At least one recognition species is a natural or unnatural, single-stranded or double-stranded nucleic acid and each further recognition species is a synthetic substance, different natural

substance or different natural substance derivative other than a nucleic acid, preferably an antibody or an antibody derivative.

6. The first recognition species and each further recognition species is a natural or unnatural, single-stranded or double-stranded nucleic acid or alternatively a synthetic substance, different natural substance or different natural substance derivative other than a nucleic acid, preferably an antibody or an antibody derivative.
7. At least one recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and another natural or unnatural, single-stranded or double-stranded nucleic acid.
8. At least one recognition species is a hybrid of a synthetic substance, a natural substance or a natural substance derivative and another synthetic substance, another natural substance or another natural substance derivative.
9. At least one recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and a synthetic substance, a different natural substance or a different natural substance derivative other than a nucleic acid, preferably an antibody or antibody derivative.
10. A first recognition species is a natural or unnatural, single-stranded or double-stranded nucleic acid, a second recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and a synthetic substance, a natural substance or a natural substance derivative, preferably an antibody or antibody derivative.
11. A first recognition species is a natural or unnatural, single-stranded or double-stranded nucleic acid, a second recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and another natural

or unnatural single-stranded or double-stranded nucleic acid, and the third recognition species is a further different natural or unnatural, single-stranded or double-stranded nucleic acid.

12. A first recognition species is a synthetic substance, a natural substance or a natural substance derivative, preferably an antibody or antibody derivative, a second recognition species is a hybrid of a synthetic substance, a natural substance or a natural substance derivative, preferably an antibody or antibody derivative, and another synthetic substance, another natural substance or another natural substance derivative, preferably another antibody or antibody derivative, and a third recognition species is a further different synthetic substance, a further different natural substance or a further different natural substance derivative, preferably a further different antibody or a further different antibody derivative.

Another subject of the present invention is a test system comprising at least two recognition species, which recognize at least two different markers with formation of a complex, preferably the recognition species or markers already described above. In a preferred embodiment, at least one recognition species is immobilized on a support, such as preferably already described above in greater detail.

The test system according to the invention can be employed in the following preferred embodiments:

1. At least one recognition species is a natural or unnatural, single-stranded or double-stranded nucleic acid and at least one other recognition species is another natural or unnatural, single-stranded or double-stranded nucleic acid.
2. At least one recognition species is a synthetic substance, a different natural substance or a different natural substance derivative other than a nucleic acid, preferably an antibody or antibody

derivative, and at least one other recognition species is another synthetic substance, different natural substance or different natural substance derivative other than a nucleic acid, preferably an antibody or antibody derivative.

3. At least one recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and a synthetic substance, different natural substance or different natural substance derivative other than a nucleic acid, preferably an antibody or antibody derivative.
4. At least one recognition species is a hybrid of a natural or unnatural, single-stranded or double-stranded nucleic acid and another natural or unnatural single-stranded or double-stranded nucleic acid.
5. At least one recognition species is a hybrid of a synthetic substance, different natural substance or different natural substance derivative other than a nucleic acid, preferably an antibody or antibody derivative, and another synthetic substance, different natural substance or different natural substance derivative other than a nucleic acid, preferably an antibody or antibody derivative.

The test system according to the invention can be produced, for example, by assembling the recognition species necessary for the individual embodiments, or by immobilizing at least one recognition species on a support, such as preferably already described above, by the process generally known to the person skilled in the art.

The test system according to the invention can be employed in the detection process according to the invention, as described in greater detail above. In particular, it is used for the detection of the presence and/or absence of at least two different markers in a sample. It is preferably present in the form of a diagnostic or an analyte. It is therefore used, in particular, for the detection of disorders or

for environmental analysis, in particular for the detection of toxins and/or allergens.

The following figures and examples are intended to describe the invention in greater detail, without restricting it.

DESCRIPTION OF THE FIGURES

Fig. 1 shows schematically the detection of two analytes (A and B) in an assay in the immobilized embodiment.

Fig. 2 shows schematically the detection of two analytes (antigens A and B) in an assay in the immobilized embodiment.

Fig. 3 shows schematically the detection of two analytes (nucleic acid A and B) in an assay in the immobilized embodiment.

Fig. 4 shows schematically the complex of markers and recognition species according to Example 1.

Fig. 5 shows schematically the complex of markers and recognition species according to Example 2.

EXAMPLES

Simultaneous detection of a deoxyribonucleic acid and of a labelled **antibody/antigen**

Starting compounds:

The reagents needed for the example, such as Texas Red®-labelled oligonucleotide conjugate (24-mer DNA; Interactiva; DNA 1), a biotinylated oligonucleotide conjugate (24-mer DNA; Interactiva; DNA 3); a synthetic oligonucleotide (57-mer DNA; Interactiva; DNA 2), which has sequences complementary to the two other DNAs, streptavidin-conjugated anti-human IgG $F(ab')_2$ (goat; Rockland) and a fluorescein-labelled human IgG $F(ab')_2$ fragment (Rockland) as antigen, are all commercially obtainable

Sub A1

Reagent	Specification
DNA 1 (recognition species 1)	Texas Red-5′-AAA-TGC-ATG-TCG-TCG-TGA-TGT-AAA-3′
DNA 2 (marker 1)	5′-ATT-GTC-ATA-ATC-ATC-TTG-AGA-CGC-TTT-TTT-TTT-TTT-ACA-TCA-CGA-CGA-CAT-GCA-TTT-3′
DNA 3 (recognition species 2)	Biotin-5′-GCG-TCT-CAA-CAT-GAT-TAT-GAC-AAT-3′
Antibodies (recognition species 3	Streptavidin-conjugated anti-human IgG $F(ab')_2$ (goat)
Antigen (marker 2)	Fluorescein-labelled human IgG $F(ab')_2$ fragment

Table 1: Recognition species and markers used.

Example 1

1 nmol of Texas Red-labelled oligonucleotide conjugate (DNA 1), 1 nmol of biotin-labelled oligonucleotide conjugate (DNA 3) and one pmol of the 57-mer oligonucleotide (DNA 2) were taken up in 150 µl of hybridization buffer (5 × SSC, 0.02% SDS) in each case. The constituents were heated at 60°C for 30 min, mixed with one another and incubated at 37°C for 3 h. They were allowed to cool to room temperature (RT), 1 nmol of the streptavidin anti-human IgG $F(ab')_2$ conjugate and 1 nmol of the fluorescein-labelled IgG $F(ab')_2$ fragment were added and the mixture was allowed to stand at RT overnight. The complex formed was detected by means of a yellow band in a non-denaturing gel (15% strength TEB gel, BioRad).

Example 2

1 nmol of biotin-labelled oligonucleotide conjugate (DNA 1), 1 nmol of biotin-labelled oligonucleotide conjugate (DNA 3) and one pmol of the 57-mer oligonucleotide (DNA 2) were taken up in 150 µl of hybridization buffer (5 × SSC, 0.02% SDS) in each

case. The constituents were heated at 60°C for 5 min, mixed with one another and incubated at 37°C for 3 h. The solution was allowed to cool to room temperature (RT) and added to a streptavidin-coated microtitre plate (BIOTEZ, order No. 040298920). The supernatant solution was removed by pipette and the support was washed 5× with 500 µl of 0.9% NaCl solution. 200 µl of a solution of 200 µl of streptavidin anti-human IgG $F(ab')_2$ (goat) solution (1.6 mg/ml) preincubated at RT for 2 h and 40 µl of the fluorescein-labelled IgG $F(ab')_2$ fragment solution (5.0 mg/ml) were then added and the mixture was incubated at RT for 1-2 h. The supernatant solution was in turn removed by pipette and the support was washed 5× with 500 µl of 0.9% NaCl solution. The formation of the complex was detected by measuring the fluorescence of the fluorescein ($\lambda_{max,A}$: 494 nm, $\lambda_{max,E}$: 525 nm).

Sub A2

Reagent	**Specification**
DNA 1′ (recognition species 1)	Biotin-5′-AAA-TGC-ATG-TCG-TCG-TGA-TGT-AAA-3′
DNA 2 (marker 1)	5′-ATT-GTC-ATA-ATC-ATC-TTG-AGA-CGC-TTT-TTT-TTT-TTT-ACA-TCA-CGA-CGA-CAT-GCA-TTT-3′
DNA 3 (recognition species 2)	Biotin-5′-GCG-TCT-CAA-CAT-GAT-TAT-GAC-AAT-3′ a
Antibodies (recognition species 3	Streptavidin-conjugated anti-human IgG $F(ab')_2$ (goat)
Antigen (marker 2)	Fluorescein-labelled human IgG $F(ab')_2$ fragment

Table 2: Recognition species and markers used.

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this invention. Thus, it is intended that the present invention cover such

modifications and variations, provided they come within the scope of the appended claims and their equivalents.

Priority application DE 198 59 912.9, filed December 23, 1998, including the specification, drawings, claims and abstract, is hereby incorporated by reference. All publications cited herein are incorporated in their entireties by reference.